Contribution of myeloid and lymphoid host cells to the curative outcome of mouse sarcoma treatment by photodynamic therapy

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Abstract

Selective depletion or inactivation of specific myeloid populations (neutrophils, macrophages) and lymphoid populations (helper T cells, cytolytic T cells) in EMT6 sarcoma-bearing mice was used to determine the contribution of each of these host immune cell types to the curative outcome of Photofrin-based photodynamic therapy (PDT). Immunodepletion of neutrophils and cytolytic T cells initiated immediately after PDT resulted in a marked reduction in PDT-mediated tumor cures. Significant reduction in the cures of EMT6 tumors was also achieved by immunodepletion of helper T cells and inactivation of macrophages by silica treatment. The initial tumor ablation by PDT was not affected by any of the above depletion treatments. These results provide direct evidence that the contribution of neutrophils, macrophages and T lymphocytes is essential for the maintenance of long-term control of PDT-treated tumors. © 1999 Published by Elsevier Science Ltd. All rights reserved.

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1. Introduction

Eradication of solid cancers by photodynamic therapy (PDT) is achieved by an integrated engagement of anti-tumor effects induced in the parenchyma, vascular network and bed of treated tumors [4,9]. In addition to direct lethal effects induced by photooxidative damage to vital cell targets, the contributing secondary tumoricidal effects include: (i) ischemic cell death subsequent to vascular damage causing microvascular collapse; (ii) ischemia-reperfusion injury; (iii) cytocidal action of activated inflammatory cells; (iv) anti-tumor immune response. Common to all of these secondary effects, which manifest as the mobilization of the host to participate in the destruc-

tion of PDT-treated tumors, is a strong inflammatory reaction [4,11].

Photooxidative lesions of membranous lipids underlie the PDT-elicited inflammatory cellular damage, induced by the extensive release of lipid degradation fragments and metabolites of arachidonic acid [11]. These products, together with mediators such as histamine and serotonin released from PDT-damaged vasculature, are potent initiators of inflammation, which is a dominating event in the anti-tumor effect of PDT. The major hallmark of the PDT-induced local acute inflammatory reaction is a massive invasion of myeloid inflammatory cells into the treated tumor. This is a regulated process, characterized by a sequential arrival of neutrophils, mast cells and monocytes/macrophages [8,16,17]. These cells were found to be highly activated and engaged in tumoricidal activity [17]. In PDT-treated tumor-

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bearing rats, the levels of neutrophils in peripheral blood were reported to increase and peak around 8 h post light exposure [3]. Increasing the numbers of circulating neutrophils in these animals (using a treatment with granulocyte colony-stimulating factor) was found to improve the response of tumors to PDT [2]. Selective enhancement of macrophage activity in tumor-bearing mice by treatment with the macrophage-activating factor DBPMAF was shown to markedly enhance the curative effect of PDT [15]. Activation of myeloid cells with granulocyte macrophage-activating factor (GM-CSF) was also demonstrated to potentiate the PDT-mediated tumor cures [18].

On the other hand, the activity of host lymphoid populations was shown to be essential for preventing the recurrence of PDT-treated mouse EMT6 sarcomas [14]. Only initial ablation, but not permanent cures of EMT6 tumors growing in scid or nude mice (which lack T and B lymphocyte activity), were obtained with Photofrin-based PDT treatment that is fully curative against EMT6 tumors growing in immunocompetent BALB/c mice. The curative effect was restored in scid mice whose bone marrow was reconstituted by transplant from BALB/c mice [14]. The generation of immune memory cells sensitized to PDT-treated tumors was also demonstrated [11].

The above evidence demonstrates that various types of inflammatory and immune effector cells participate in the responses triggered during and after treatment of tumors by PDT. In the present study, selective depletion or inactivation of specific myeloid and lymphoid cell populations was used to determine their contribution to the curative outcome of PDT in the treatment of mouse tumors.

2. Materials and methods

The EMT6 mammary sarcoma [22] was maintained in syngeneic BALB/c mice by bi-weekly intramuscular transplantation. For experiments, 1×10^6 cells were implanted subcutaneously into a lower dorsal region. The tumors reached the size for PDT treatment (6–8 mm in largest diameter) in 7–9 days after inoculation. Only female mice 7–9 weeks of age at the time of PDT treatment were used.

Individual treatment groups consisted of eight

tumor-bearing mice. Photofrin (porfimer sodium; provided by QLT PhotoTherapeutics Inc., Vancouver, B.C., Canada) was administered at a dose of 10 mg/kg injected intravenously (i.v.) 24 h before treatment with 630 ± 10 nm light. The monodirectional beam from a tunable light source based on a 1 kW xenon bulb (Model A5000; Photon Technology International) delivered through a 5 mm-core diameter liquid light guide (2000 A; Luminex, Munich, Germany) illuminated the area encompassing the tumor and 1 mm of surrounding normal tissue of mice restrained unanaesthetized in lead holders exposing their backs. The light dose delivered to tumors was 110 J/cm^2, with a power density of 120–130 mW/cm^2.

The evaluation of response to investigational treatments was based on tumor cure/recurrence. No sign of tumor at the end of the observation period (90 days) qualified as a cure. Statistical analysis of the data was based on the long-rank test.

A series of hybridoma cultures were used for the production of monoclonal antibodies (mAbs) needed for immunodepletion or as isotype controls. Hybridomas producing mAbs reacting with mouse GR-1 antigen (Ly-6G) expressed on mouse granulocytes (clone RB6-8C5, rat IgG2b) were used with the permission of Dr. Gerald J. Spangrude (Department of Medicine, University of Utah). The hybridoma cell lines obtained from ATTC were: GK1.5 (TIB 205, producing rat IgG2b against mouse CD4), 3.155 (TIB 211, producing rat IgM against mouse CD8), PC 61 5.3 (TIB 222, producing rat IgG1 against mouse CD25), RA3-3A1/6.1 (TIB 146, producing rat IgM against B220 antigen on mouse B cells) and Cy34.1.2 (TIB 163, producing IgG1 reacting with mouse B lymphocyte differentiation antigen CD22 on BALB/c and other Lyb-8.2 allotype strains). These hybridomas were maintained in DMEM medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, UT). Before antibody collection, the cultures were transferred into serum-free and protein-free hybridoma medium (Sigma S-8284). Two days later, the media supernatants were harvested and the secreted mAbs concentrated to 2–4 mg/ml using 50 000 MW concentrating filters (Biomax-50, Millipore Corporation, Bedford, MA). Rat IgG2b raised against murine macrophage scavenger receptor, produced by hybridoma clone
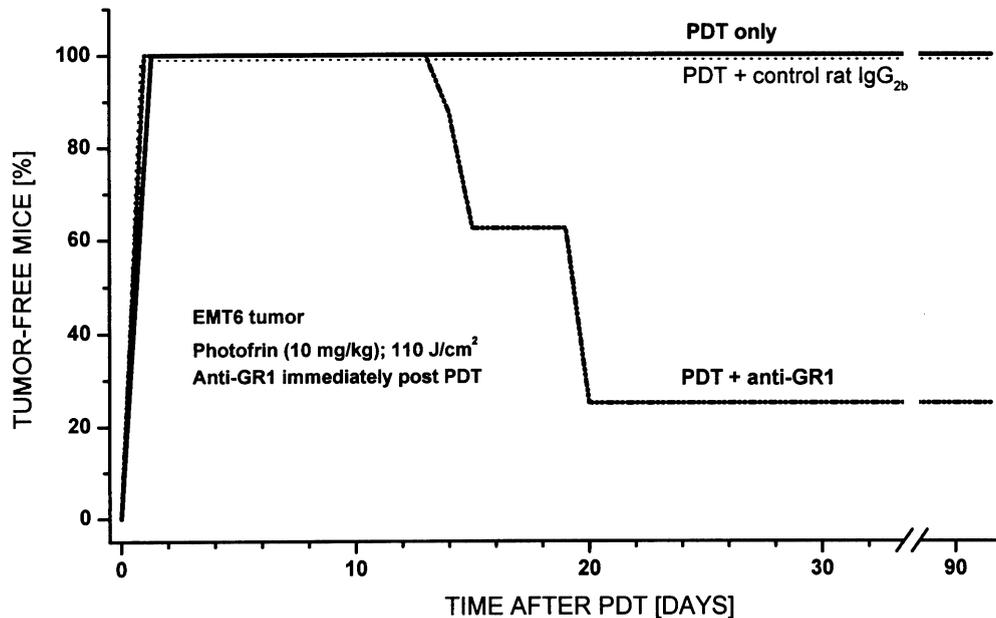
Fig. 1. The effect of neutrophil depletion on the response of EMT6 tumors to Photofrin-based PDT. Tumor-bearing BALB/c mice were given Photofrin (10 mg/kg i.v.) and 24 h later the tumors were exposed to 110 J/cm^2 of 630 $\pm$ 10 nm light. For neutrophil depletion, the mice were treated with rat IgG2b mAbs reacting with GR-1 antigen, which was injected i.v. (5 mg/kg) immediately after the termination of light treatment. The same treatment with rat IgG2b mAbs raised against the macrophage scavenger receptor served as an isotype control for anti-GR-1. The mice were observed afterwards for signs of tumor recurrence. The difference in cure rates between the PDT-only (or PDT plus isotype control) group and the PDT plus anti-GR-1 group is statistically significant ($P < 0.005$).

2F8, was obtained purified in azide-free form from Cedarlane Laboratories Ltd. (Hornby, Ont., Canada). All mAbs were administered to mice i.v. at a dose of 5 mg/kg given immediately after termination of photodynamic light treatment.

Silica (silicon dioxide), purchased from Sigma, was used for inactivation of macrophages. The suspension of silica in 5% dextrose used for intraperitoneal (i.p.) injection into mice (1 g/kg) was sonicated prior to use.

3. Results

Depletion of neutrophils from the blood of mice can be effectively achieved by in vivo administration of anti-GR-1 mAbs [21]. Differential counts of peripheral blood smears of treated mice revealed that >95% of neutrophils are depleted 30 min after the antibody i.v. injection (the effect was mostly completed within 15 min), and the levels of these cells remain very low

for at least 1 day (data not shown). With some tumor models, neutrophil depletion may inhibit tumor growth and/or reduce the recruitment of tumor-associated macrophages [21]. We have looked for these changes with the EMT6 tumor model, but no significant differences were detected. The effect of neutrophil depletion on the response of EMT6 tumors to Photofrin-based PDT is shown in Fig. 1. The PDT treatment chosen was fully curative, i.e. there was no sign of regrowth of any tumor treated by PDT alone. However, i.v. injection of anti-GR-1 (5 mg/kg) immediately after completion of photodynamic light treatment resulted in a drastic reduction of the curative effect, as evidenced by the recurrence of most of the EMT6 tumors between 2 and 3 weeks after PDT. The initial tumor ablation induced within 24 h post-PDT appeared not to be affected by neutrophil depletion. No effect on PDT response was detectable in tumor-bearing mice injected immediately after PDT with the same type of immunoglobulin (rat IgG$_{2b}$) recognizing a different antigen (mouse macro-

phage scavenger receptor), which confirms the specificity of anti-GR-1 treatment.

Selective depletion of macrophages in mice is not achievable, but exposure to silica particles in vitro and in vivo is known to induce lysis of some of these cells and selective impairment of function in others [6,19]. The effect of silica administered to mice immediately after photodynamic light treatment of EMT6 tumors is shown in Fig. 2. It is evident that this treatment aimed at macrophage inactivation markedly decreased the curative response of tumors to PDT, while having no obvious effect on the initial ablation of PDT-treated tumors.

In further experiments, selective immunodepletion was used to investigate the importance of specific lymphoid populations in the curative response of tumors to PDT. The mAbs 3.155 (anti-CD8) and GK1.5 (anti-CD4) used for in vivo depletion of cytolytic and helper T lymphocytes, respectively, were proven effective for this purpose in previously published studies [10,23]. Our flow cytometry analysis of blood leukocytes from anti-CD8 and anti-CD4 treated BALB/c mice (both 5 mg/kg i.v.)

showed less than 1% of targeted populations remaining at 1 h post i.v. injection with both of these antibodies (data not shown). Levels of target populations in lymphoid tissues (spleen, lymph nodes) were also severely depressed.

The effect of such depletion in EMT6 tumor-bearing mice initiated immediately after completion of photodynamic light treatment is shown in Figs. 3 and 4. Similar to neutrophil depletion (Fig. 1), the anti-CD8 mAbs administered immediately after PDT showed no demonstrable effect on the initial ablation of PDT-treated tumors, but the eventual cure rate decreased from 100% to around 50% (Fig. 3). The equivalent treatment with the control antibody of the same immunoglobulin class (rat IgM; raised against B220 membrane glycoprotein), not blocking the activity of target cells [1] showed no demonstrable interference with PDT-mediated tumor cures. The depletion of $CD4^+$ T lymphocytes with GK1.5 antibody (rat IgG2b) initiated immediately after PDT also reduced the cure rate of EMT6 tumors in a similar fashion as $CD8^+$ cell depletion, even though the effect was less pronounced (but statistically significant)
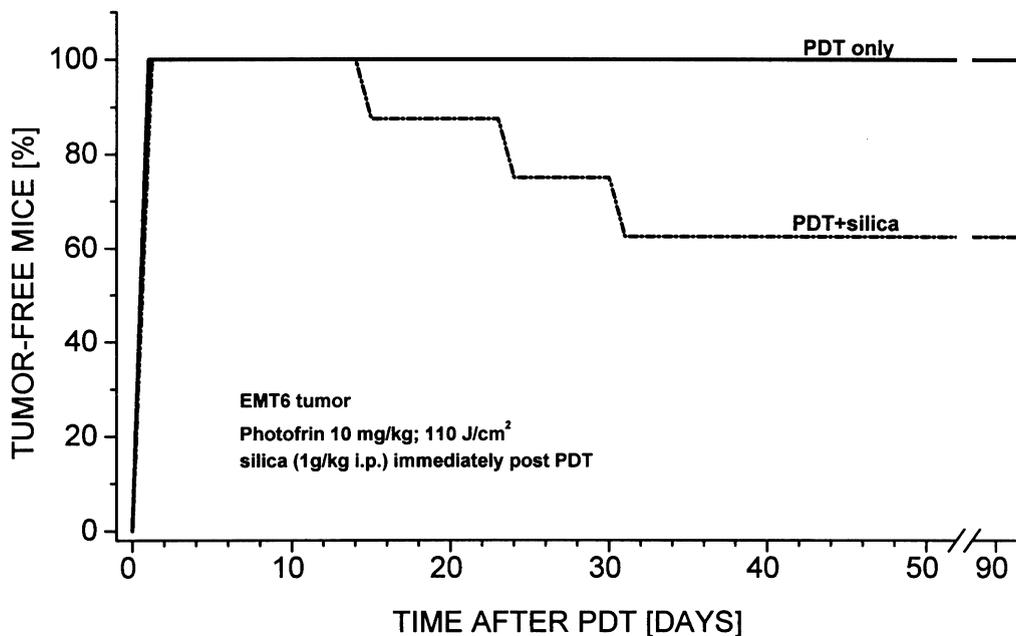

Fig. 2. The effect of macrophage inactivation with silica on the response of EMT6 tumors to Photofrin-based PDT. Tumor-bearing mice were treated with silica (1 g/kg i.p.) immediately after photodynamic light treatment. The PDT protocol and other details were as described in Fig. 1. The difference in cure rates between PDT-only and PDT plus silica groups is statistically significant ($P < 0.01$).
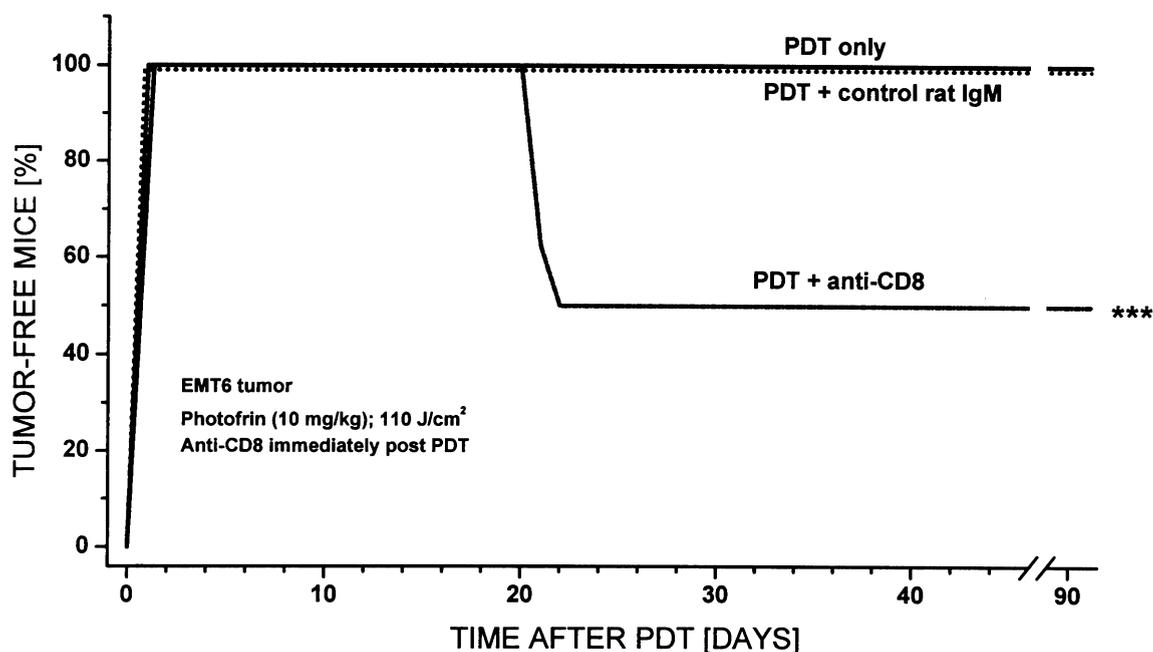
Fig. 3. The role of cytolytic T lymphocytes in the response of EMT6 tumors to Photofrin-based PDT. Tumor-bearing mice were injected with either anti-CD8 mAbs or anti-B220 mAbs (IgM isotype controls for anti-CD8). The mAbs (each at 5 mg/kg) were administered i.v. immediately after photodynamic light treatment. The PDT treatment and other details were as described in Fig. 1. The statistical significance for the difference in response between PDT-only and PDT plus anti-CD8 treatment group was $P < 0.005$.

(Fig. 4). The control treatment for rat IgG2b isotype is depicted in Fig. 1.

The PDT-mediated cure rate of EMT6 tumors was also reduced by the administration of mAbs raised against the CD25 antigen, i.e. receptor for interleukin-2 (IL-2), as depicted in Fig. 4. The antibody used inhibits binding of IL-2- and IL-2-dependent proliferation [25]. For reasons of clarity, the results of treatment with the isotype control antibody (IgG1 recognizing CD22 antigen) are not shown. This antibody, which does not measurably impair functions of targeted B cells [24], had not visibly affected tumor cures. Also depicted in Fig. 4 is the effect of the combined treatment with anti-CD4 and anti-CD25 antibodies performed immediately after PDT. The degree of reduction in PDT-mediated tumor cures with this combination was similar to that obtained with anti-CD8 alone. No additional reduction in cures of PDT-treated tumors was achieved with anti-CD8 plus anti-CD4 or anti-CD8 plus anti-CD25 combinations.

Without PDT, neither silica nor antibodies examined in this study produced any detectable effect on tumor growth. In the case of tumor recurrence, the rate of tumor growth in mice from PDT-plus-depletion groups was not different than that observed with tumors recurring after non-curative PDT-only doses.

4. Discussion

In this study, we have chosen not to target the activity of host immune cells before PDT. Any manipulation of these cells may affect their orderly association with growing tumors, or interfere with the photosensitizer localization and cause other changes that may alter the initial PDT insult. It is possible that repeated treatments with depleting antibodies, or with silica, performed before and after PDT, may have a more profound impact on the tumor cure rate than the single treatments. Nevertheless, the reductions in cure rate of PDT-treated
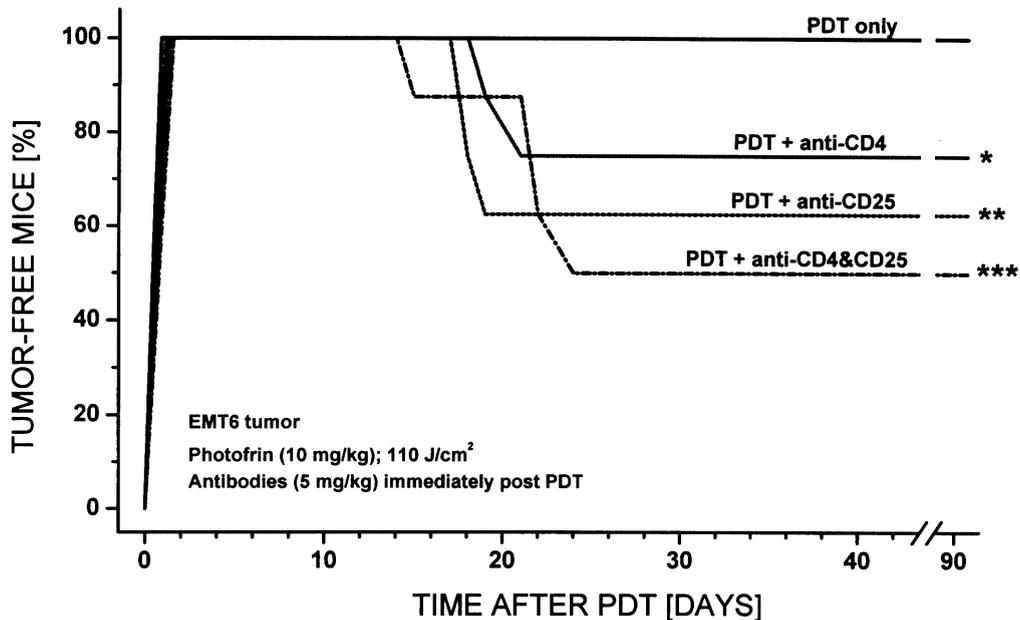
Fig. 4. The effect of helper T cell depletion or blocking of IL-2 receptor on the response of EMT6 tumors to Photofrin-based PDT. Immediately after photodynamic light treatment, tumor-bearing mice were administered ether anti-CD4 or anti-CD25 (both at 5 mg/kg i.v.), or their combination. The PDT treatment and other details were as described in Fig. 1. The statistical significance for the difference in response between PDT-only and other treatment groups: *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$.

tumors attained by single treatment protocols depicted in this report are sufficient to demonstrate that the activities of neutrophils, macrophages, helper and cytolytic T lymphocytes are directly responsible for the curative outcome of EMT6 tumor treatment by Photofrin-based PDT.

Neutrophils, macrophages, helper and cytolytic T lymphocytes activated by PDT can be expected to contribute to tumor cures in different ways. Neutrophils were found to invade rapidly after the onset of photodynamic light exposure and accumulate in large numbers in PDT-treated tumors [8,16,17]. This can be attributed to strong chemoattracting signals released due to PDT-inflicted inflammatory damage [11]. Another event associated with massive invasion of neutrophils is ischemia-reperfusion injury, which appears also to be induced in PDT-treated tumors [12]. Activated neutrophils are capable of inflicting considerable damage to tumor vasculature and (upon extravasation) to perivascular regions of tumor parenchyma. Release of a variety of potent tissue-destructive enzymes and radicals by errant neutrophils is well documented [7]. The results obtained with

neutrophil depletion (Fig. 1) are in accordance with the finding in a rat rhabdomyosarcoma model [2], that PDT-induced delay in tumor growth was diminished in hosts treated with anti-rat neutrophil serum.

Macrophages that participate in anti-tumor effects elicited by PDT may be either those already present in tumors before PDT (if not inactivated by photo-toxic effects), or newly infiltrated cells attracted to the tumor by PDT-induced signals [13,17]. Macrophages isolated from mouse SCCVII tumors at 2 h post PDT were shown to exhibit almost five times greater tumoricidal activity than those from non-treated SCCVII tumors [17]. In addition to engaging in tumor cell killing as non-specific immune effector cells, macrophages were suggested to function as professional antigen-presenting cells (APCs) in PDT-treated tumors [11]. In most cases, a large majority of cancer cells (>90%) will disintegrate (going through either necrotic or apoptotic death) within the first 10 h following Photofrin-based PDT. Large amounts of debris of many dead cancer cells generated in this way within a short time interval, will compel macrophages to engage in the

ingestion of tumor cell remnants. Acting as APCs, and directed by PDT-induced stimulatory and accessory signaling, these macrophages may process peptides from ingested cancer cells and present them on their membranes in the context of MHC molecules. This will enable the recognition of tumor antigens by T lymphocytes, setting the stage for the development of a tumor-specific immune response. Silica particles administered in vivo were reported to be particularly effective in blocking the antigen-presenting function of macrophages [6].

The results of immunodepletion experiments suggest that both helper and cytotoxic T cell populations, as well as IL-2 signaling, participate in the development of an immune response induced by the PDT treatment of EMT6 tumors. The fact that the depletion of $CD8^+$ cells impaired the curative effect more markedly than depletion of $CD4^+$ cells may indicate that cytolytic T lymphocytes are mainly responsible for eliminating cancer cells surviving the initial phototoxic effects of PDT, while helper T lymphocytes play a supportive role. The nature of participation of these two T cell populations in the anti-tumor response may be determined by the type of tumor antigen recognized by the immune system. Further investigation is needed to establish whether in certain other tumors the role of either $CD4^+$ or $CD8^+$ lymphocyte populations may not be obligatory for the development of PDT-induced immune response.

Release of various cytokines, including IL-1β, IL-2, IL-6, G-CSF and TNF-α, has been observed or implicated following treatment of tumors by PDT [3,5,8,20]. A future extension of this study should examine how the production of these and other cytokines/chemokines is affected by the depletion of specific host immune populations.

In summary, this study provides direct evidence for the contribution of the cellular arm of the host immune system to tumor cure attained by PDT. The results demonstrate that the participation of various types of non-specific and specific immune effector cells is not just a secondary reaction to more relevant events, but is actually required for the successful outcome of this cancer therapy. The activity of these cells may not be critical for achieving the initial ablation of PDT-treated tumors, but is a key determinant for the maintenance of long-term tumor control.

References

[1] R.L. Coffman, I.L. Weissman, B220: a B cell-specific member of the T200 glycoprotein family, Nature 289 (1981) 681–683.

[2] W.J.A. de Wree, M.C. Essers, H.S. DeBrujin, W.M. Star, J.F. Koster, W. Sluiter, Evidence for an important role of neutrophils in the efficacy of photodynamic therapy *in vivo*, Cancer Res. 56 (1996) 2908–2911.

[3] W.J.A. de Wree, M.C. Essers, J.F. Koster, W. Sluiter, Role of interleukin 1 and granulocyte colony-stimulating factor in Photofrin-based photodynamic therapy of rat rhabdomyosarcoma tumors, Cancer Res. 57 (1997) 2555–2558.

[4] T.J. Dougherty, C.J. Gomer, B.W. Henderson, G. Jori, D. Kessel, M. Korbelik, J. Moan, Q. Peng, Photodynamic therapy, J. Natl. Cancer Inst. 90 (1998) 889–905.

[5] S. Evans, W. Matthews, R. Perry, D. Fraker, J. Norton, H.I. Pass, Effect of photodynamic therapy on tumor necrosis factor production by murine macrophages, J. Natl. Cancer Inst. 82 (1990) 34–39.

[6] A.B. Frey, Role of host antigen receptor-bearing and antigen receptor-negative cells in immune response to rat adenocarcinoma 13762, J. Immunol. 156 (1996) 3841–3849.

[7] J.I. Gallin, Inflammation, in: W.E. Paul (Ed.), Fundamental Immunology, Raven Press, New York, 1989, pp. 721.

[8] S.O. Gollnick, X. Liu, B. Owczarczak, D.A. Musser, B.W. Henderson, Altered expression of interleukin 6 and interleukin 10 as a result of photodynamic therapy *in vivo*, Cancer Res. 57 (1997) 3904–3909.

[9] B.W. Henderson, T.J. Dougherty, How does photodynamic therapy work?, Photochem. Photobiol. 55 (1992) 145–157.

[10] H. Hock, M. Dorsch, T. Diamantstein, T. Blankenstein, Interleukin 7 induces $CD4^+$ T cell-dependent tumour rejection, J. Exp. Med. 174 (1991) 1291–1298.

[11] M. Korbelik, Induction of tumour immunity by photodynamic therapy, J. Clin. Laser Med. Surg. 14 (1996) 329–334.

[12] M. Korbelik, I. Cecic, H. Shibuya, The role of nitric oxide in the response of cancerous lesions to photodynamic therapy, in: S.G. Pandalai (Ed.), Recent Research Developments in Photochemistry and Photobiology, Transworld Research Network, Trivandrum (India), 1998, pp. 267.

[13] M. Korbelik, G. Krosl, Photosensitizer distribution and photosensitized damage of tumor tissues, in: H. Hönigsmann, G. Jori, A.R. Young (Eds.), The Fundamental Bases of Phototherapy, OEMF spa, Milan, 1996, pp. 229.

[14] M. Korbelik, G. Krosl, J. Krosl, G.J. Dougherty, The role of host lymphoid populations in the response of mouse EMT6

tumor to photodynamic therapy, Cancer Res. 56 (1996) 5647–5652.

[15] M. Korbelik, V.R. Naraparaju, N. Yamamoto, Macrophage-directed immunotherapy as adjuvant to photodynamic therapy of cancer, Br. J. Cancer 75 (1997) 202–207.

[16] M. Korbelik, I. Cecic, Enhancement of tumor response to photodynamic therapy by adjuvant mycobacterium cell wall treatment, J. Photochem. Photobiol. B: Biol. 44 (1998) 151–158.

[17] G. Krosl, M. Korbelik, G.J. Dougherty, Induction of immune cell infiltration into murine SCCVII tumour by Photofrin-based photodynamic therapy, Br. J. Cancer 71 (1995) 549–555.

[18] G. Krosl, M. Korbelik, G.J. Dougherty, Potentiation of photo-dynamic therapy-elicited antitumor response by localized treatment with granulocyte-macrophage colony-stimulating factor, Cancer Res. 56 (1996) 3281–3286.

[19] M.H. Levy, E.F. Wheelock, Effects of intravenous silica on immune and non-immune functions of the murine host, J. Immunol. 115 (1975) 41–48.

[20] U.O. Nseyo, R.K. Whalen, M.R. Duncan, B. Berman, S.L. Lundhal, Urinary cytokines following photodynamic therapy

for bladder cancer. A preliminary report, Urology 36 (1990) 167–171.

[21] L.A. Pekarek, B.A. Starr, A.Y. Toledano, H. Schreiber, Inhi-bition of tumour growth by elimination of granulocytes, J. Exp. Med. 181 (1995) 435–440.

[22] S.C. Rockwell, R.F. Kallman, L.F. Fajardo, Characteristics of a serially transplanted mouse mammary tumor and its tissue-culture-adapted derivative, J. Natl. Cancer Inst. 49 (1972) 735–749.

[23] M. Sarmiento, D.P. Dialynas, D.W. Lancki, K.A. Wall, M.I. Lorber, M.R. Loken, F.W. Fitch, Cloned T lymphocytes and monoclonal antibodies as probes for cell surface molecules active in T cell-mediated cytolysis, Immunol. Rev. 68 (1982) 135–169.

[24] F.W. Symington, B. Subbarao, D.E. Mosier, J. Sprent, Lyb-8.2: a new cell antigen defined and characterized with a mono-clonal antibody, Immunogenetics 16 (1982) 381–391.

[25] R.H. Zubler, J.W. Lowenthal, F. Erard, N. Hashimoto, R. Devos, H.R. MacDonald, Activated B cells express receptors for, and proliferate in response to, pure interleukin 2, J. Exp. Med. 160 (1984) 1170–1183.